

BRU1, a novel link between responses to DNA damage and epigenetic gene silencing in *Arabidopsis*

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DNA repair associated with DNA replication is important for the conservation of genomic sequence information, whereas reconstitution of chromatin after replication sustains epigenetic information. We have isolated and characterized mutations in the *BRU1* gene of *Arabidopsis* that suggest a novel link between these underlying maintenance mechanisms. *Bru1* plants are highly sensitive to genotoxic stress and show stochastic release of transcriptional gene silencing. They also show increased intrachromosomal homologous recombination and constitutively activated expression of poly (ADP-ribose) polymerase-2 (*AtPARP-2*), the induction of which is associated with elevated DNA damage. *Bru1* mutations affect the stability of heterochromatin organization but do not interfere with genome-wide DNA methylation. *BRU1* encodes a novel nuclear protein with two predicted protein–protein interaction domains. The developmental abnormalities characteristic of *bru1* mutant plants resemble those triggered by mutations in genes encoding subunits of chromatin assembly factor (CAF-1), the condensin complex, or *MRE11*. Comparison of *bru1* with these mutants indicates cooperative roles in the replication and stabilization of chromatin structure, providing a novel link between chromatin replication, epigenetic inheritance, S-phase DNA damage checkpoints, and the regulation of meristem development.

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A dynamic chromatin structure contributes to the regulation of repair and transcription of DNA templates. Chromatin components involved in both processes have been described that imply shared molecular mechanisms modulating DNA accessibility for repair and transcription (Green and Almouzni 2002). The first molecular link between transcription and DNA repair was revealed during characterization of transcription factor IIIH (TFIIH), which is required for initiation of RNA synthe-

sis by RNA polymerase II and for efficient repair of DNA through nucleotide excision (Feaver et al. 1993; Schaeffer et al. 1993; Drapkin et al. 1994; Wang et al. 1994).

Accessibility is determined by compaction of chromatin, which consists of loosely packaged, transcriptionally active euchromatin, and heterochromatin, which is condensed and transcriptionally silent and consists mainly of transposable elements and repetitive sequences. Chromatin states are inherited during DNA replication, providing a scaffold for epigenetic information that influences transcriptional gene regulation.

Several chromatin components determining heritable features of chromatin also have an influence on epigenetic regulation of gene activity and efficiency of DNA repair or genome stability. For example, SIR proteins in yeast mediate formation of a compact chromatin structure similar to heterochromatin in multicellular eukaryotes (Gross 2001) and are required for transcriptional gene silencing (TGS) and for suppression of homologous recombination of rDNA repeats (Guarente 2000). They

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are also involved in repair of DNA double-strand breaks (DSBs) by nonhomologous end joining (NHEJ; Tsukamoto et al. 1997). Thus, *SIR* mutations result in both release of TGS (Gross 2001) and increased sensitivity to DNA-damaging agents (Tsukamoto et al. 1997; Critchlow and Jackson 1998; Martin et al. 1999; Mills et al. 1999). A further requisite for accurate regulation of TGS and proper responses to genotoxic stress is the chromatin assembly factor 1 (CAF-1), which is conserved throughout eukaryotes (Kaufman and Almouzni 2000). CAF-1 interacting with PCNA (Shibahara and Stillman 1999) facilitates incorporation of histones H3 and H4 into newly synthesized DNA during S phase (Smith and Stillman 1989) and unscheduled DNA-repair synthesis (Gailard et al. 1996). Yeast mutants with defective CAF-1 subunits (*cac* mutants) are hypersensitive to UV irradiation (Kaufman et al. 1997) and unable to maintain telomeric silencing (Kaufman et al. 1997; Tchenio et al. 2001).

The replication-coupling assembly factor (RCAF; Tyler et al. 1999) also facilitates the assembly of nucleosomes onto newly replicated DNA. RCAF is a protein complex of a *Drosophila* ortholog of the anti-silencing function-1 protein (ASF1) and histones H3 and H4 (Tyler et al. 1999) that acts synergistically with CAF-1 in chromatin assembly. In yeast, both CAF-1 and ASF1/RCAF are involved in chromatin reconstitution after DNA replication and repair-coupled DNA synthesis, but their functions do not fully overlap: *cac1* and *asf1* mutations have dissimilar effects on nucleotide excision repair and homologous recombination and also differ in silencing at subtelomeric regions or mating-type loci (Tyler et al. 1999). Nevertheless, they clearly have cooperative functions, as *asf1/cac1* double mutants exhibit synergistic loss of silencing and deficiencies in DNA repair (Tyler et al. 1999).

A genetic link between DNA repair and TGS has been documented for unicellular green algae (*Chlamydomonas reinhardtii*). Transcriptionally silenced transgenes and transposable elements were activated in mutants *mut-9* and *mut-11*, both of which were hypersensitive to conditions provoking DSBs (Jeong et al. 2002). *MUT-9* encodes a putative serine/threonine protein kinase and *MUT-11* a novel protein containing a WD-40 repeat (Jeong et al. 2002). Their precise molecular functions are unknown.

Although genes involved in DNA repair or epigenetic regulation of transcription have been studied extensively in multicellular eukaryotes (Gorbunova and Levy 1999; Mittelsten Scheid and Paszkowski 2000; Hoeijmakers 2001; Martienssen and Colot 2001; Li 2002; Grewal and Moazed 2003), evidence for components linking DNA repair and epigenetic inheritance is scarce. It is reasonable to predict that such shared components exist also in higher plants and may be revealed by forward genetic approaches. Here we describe the results of two parallel searches in two different laboratories for *Arabidopsis* mutants affected in DNA-damage responses and for mutants affected in maintenance of TGS. This led to the isolation of different mutant alleles of *BRU1*, which en-

codes a novel nuclear protein. *Bru1* mutants have phenotypic similarities to *Arabidopsis* mutants in CAF-1 subunits (*fas1* and *fas2*; Kaya et al. 2001), *MRE11* (Bundock and Hooykaas 2002), and condensin genes (Siddiqui et al. 2003).

Phenotypic, genetic, and molecular characterization of *bru1*, *fas1*, *fas2*, *mre11*, and mutants in subunits of the condensin complex indicates that BRU1 is involved in epigenetic inheritance during DNA replication. Moreover, mutations in *Arabidopsis* *MRE11* or condensin subunits genes, in addition to defects in an S-phase checkpoint (Aono et al. 2002; D'Amours and Jackson 2002; Hagstrom and Meyer 2003), also compromise epigenetic regulation, as shown by release of heterochromatin-mediated TGS. Thus, our results imply a tight link between chromatin replication, postreplicative DNA repair, and an S-phase DNA-damage checkpoint.

Results

Isolation of *bru1* mutants

In a search for plant components involved in the recognition and repair of DNA damage, we screened 2500 *Arabidopsis* T3 families mutagenized by random insertion of foreign DNA (T-DNA) for individuals with elevated sensitivity to methyl methane sulfonate (MMS). MMS alkylates DNA and is considered to mimic DNA DSB damage (Schwartz 1989). One transgenic family segregated individuals unable to withstand 25 ppm of MMS, whereas wild-type plants tolerated 100 ppm (Fig. 1A). This sensitivity threshold of 25 ppm is significantly (two- to threefold) lower than for previously characterized *Arabidopsis* mutants hypersensitive to MMS (Mengiste et al. 1999; Ulm et al. 2001; Bundock and Hooykaas 2002; Garcia et al. 2003). The sensitivity segregates as a recessive monogenic trait. The homozygous mutant plants are also hypersensitive to bleomycin (inducer of DSBs; Fig. 1B), mitomycin C (cross-linking of DNA strands; Fig. 1C), and UV-C irradiation (Fig. 1D). Importantly, the sensitivity of the mutant seems to be restricted to genotoxic stresses. Mutant plants do not differ from wild-type plants in sensitivity to abiotic stresses such as high salinity, elevated osmolarity, or oxidative stress (data not shown). The mutant was named *brushy1* (*bru1*) because of its morphological abnormalities, and this particular mutant allele was designated *bru1-1*. The most prominent morphological alterations of plants homozygous for *bru1-1* are retarded growth of primary roots, distorted phyllotaxy (resulting in an irregular branching pattern), and fasciation (thick and flattened stems and fused organs; Fig. 2A–F). The degree of fasciation varies between individual *bru1-1* mutant plants and even between neighboring branches of the same plant (Fig. 2D). Fasciation affects stems, lateral shoots, and even siliques, which arise close together and form brush-like structures (Fig. 2C,F). There is no progression in severity of the phenotypes over subsequent generations. Flowering time also varies among individuals, some of which show a prolonged vegetative phase, particularly under short-day conditions (data not shown).

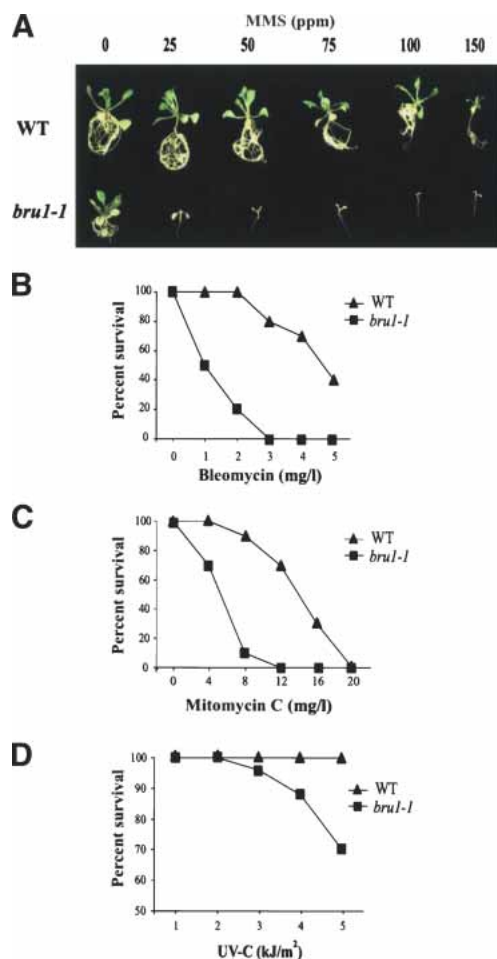


Figure 1. Sensitivity of *bru1* to genotoxic stress. Representative seedlings (A) and the survival rate (B–D) after each treatment are displayed. Levels of resistance of wild-type Ws (WT) and *bru1-1* to MMS (A), bleomycin (B), mitomycin C (C), and UV-C (D). Ten (A–C) or fifty (D) seedlings were tested in each treatment.

An independent screen after ethyl methane sulfonate (EMS) mutagenesis for mutations that release TGS at a transgenic locus encoding the luciferase (*Luc*) marker gene yielded a mutant that morphologically closely resembles *bru1-1*. Genetic analysis and sequencing of the affected *BRU1* gene in the two mutants showed them to be allelic, and the second mutant allele was designated *bru1-2*.

A third mutant allele (*bru1-3*) was identified in a collection of T-DNA insertion lines from the Torrey Mesa Research Institute (<http://www.tmri.org>). Although some aspects of the altered morphology of *bru1-3* resemble *bru1-1* and *bru1-2* phenotypes, this mutant allele is further characterized by severe dwarfing and very low seed set (Fig. 2G). This strong phenotype of *bru1-3*, which is not observed for the other two alleles, suggests that *bru1-1* and *bru1-2* are likely partial loss-of-function alleles. Extreme sensitivity to various genotoxic stresses, as initially observed for *bru1-1*, is also characteristic of

bru1-2 and *bru1-3* mutant plants (data not shown). The segregation of all observed mutant traits indicates that *bru1-2* and *bru1-3* are also recessive.

The BRU1 gene encodes a novel nuclear protein

Because the T-DNA residing in the *bru1-1* strain was not genetically linked with the mutation, the *BRU1* gene was identified by map-based cloning (data not shown). Sequence analysis of *bru1-2* and phenotypic characterization of the putative insertion allele *bru1-3* assigned all three alleles to the *BRU1* gene (Fig. 3A,B). *BRU1* is located in the upper arm of chromosome 3 and encodes a putative open reading frame of 1311 amino acids (At3g18730; GenBank accession no. AY560347). The predicted *BRU1* protein shows no obvious similarity to any protein with an assigned function present in the public databases. However, *BRU1* contains two conserved domains involved in protein–protein interaction, namely tetratricopeptide repeats (TPRs; Lamb et al. 1995; Blatch and Lassle 1999) and leucine-rich repeats (LRRs; Kobe and Deisenhofer 1994), located in the N- and C-terminal parts of the protein, respectively (Fig. 3B). *BRU1* also includes a predicted coiled-coil region and a leucine zipper motif that can potentially interact with DNA (Fig. 3B). There is no other gene related to *BRU1* present in *Arabidopsis*. There is a putative *BRU1* homolog in rice (chromosome 2, BAC clone; AP004095) that shows 45% identity and 62% similarity over the entire coding region. However, there are no obvious *BRU1* homologs in animals or fungi.

The *bru1-1* allele has a 31-bp deletion removing the junction between the 10th intron and the 11th exon, causing mis-splicing and thereby a frame-shift, which results in truncation of the C-terminal LRR domain (Fig.

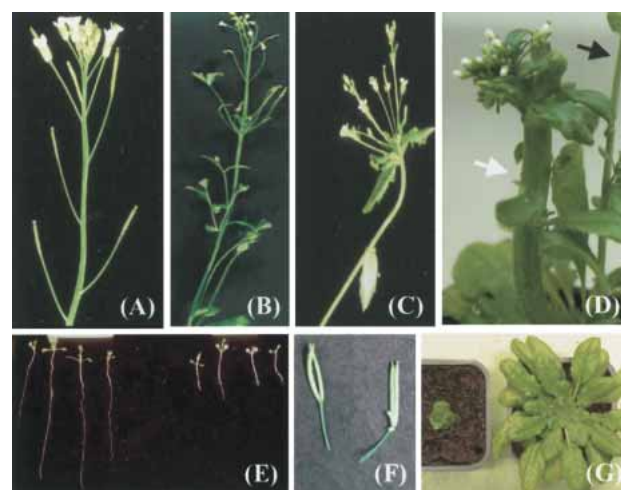


Figure 2. Developmental aberrations in *bru1*. Branching pattern of wild-type (A) and *bru1-1* (B,C) plants. (D) Fasciated (white arrow) and normal (black arrow) stems from the same *bru1-1* plant. (E) Root growth of seedlings of wild-type Ws (left) and *bru1-1* (right). (F) Fused siliques in *bru1-1*. (G) *bru1-3* mutant allele (left) and wild type (right).

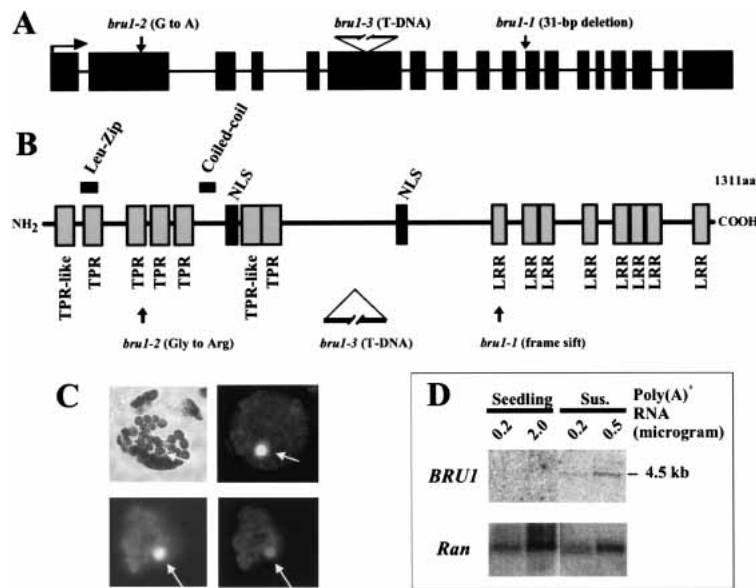


Figure 3. The *BRU1* gene encodes a novel nuclear protein. (A) Structure of the *BRU1* gene and its mutant alleles (exons are marked as black rectangles, introns as lines, and translation start as horizontal arrow). Positions of mutations are indicated. (B) Structure of the *BRU1* protein. Predicted functional motifs and positions of mutations are indicated. (C) Nuclear localization of *BRU1*–GFP fusion protein, showing a bright-field image of a transformed protoplast (top left) and a dark-field image of GFP fluorescence (top right, no Triton X-100 treatment). Below images of DAPI-stained nuclei (bottom left) and GFP fluorescence (bottom right) in the presence of Triton X-100. Nuclei are indicated by arrows. (D) Northern blot analysis of *BRU1* mRNA. Lanes with poly(A)⁺ RNA from seedlings and suspension culture cells (Sus.) are marked. RNA was hybridized with probes for *BRU1* and *AtRanBP1a* (*Ran*; Haizel et al. 1997) as a loading control.

3A,B). The *bru1-2* mutation produces a single nucleotide substitution (G to A) and consequent amino acid exchange (Gly into Arg) in the N-terminal TPR region. Interestingly, the change in *bru1-2* is at the amino acid in position 27 that is crucial for the antiparallel helical structure of the 34-amino acid-long TPR unit (Blatch and Lasse 1999). In *bru1-3*, the T-DNA insertion disrupts the linker section between the TPR and LRR regions (Fig. 3B).

As mutations in *BRU1* render the plant extremely sensitive to DNA-damaging treatment, it was to be expected that *BRU1* performs its function in the nucleus. Indeed, *BRU1* contains two predicted nuclear localization signals (Fig. 3B). To verify the subcellular localization of *BRU1*, we constructed an N-terminal protein fusion of *BRU1* with the green fluorescent protein (GFP). The *BRU1*–GFP fusion protein, when expressed in *Nicotiana plumbaginifolia* protoplasts, was found exclusively in the nucleus (Fig. 3C).

The *BRU1* mRNA (4.5 kb) is not readily detectable by Northern blot analysis of various plant tissues, but it is clearly more abundant in suspension culture cells (Fig. 3D). Moreover, a tobacco homolog of *BRU1* is expressed preferentially in S phase (T. Suzuki, pers. comm.).

Frequency of intra-chromosomal homologous recombination is elevated in *bru1*

Because *bru1* mutants are sensitive to treatments that increase levels of DSBs, and DSBs are repaired by either homologous recombination (HR) or nonhomologous end joining (NHEJ), we examined whether *bru1* is altered in either mechanism. To determine the level of HR, *bru1-1* was crossed to a transgenic line containing a chromosomally integrated recombination substrate consisting of two overlapping parts of the β -glucuronidase (GUS) transgene (Fig. 4A; Swoboda et al. 1994). An intrachro-

mosomal recombination event between the homologous regions of the incomplete transgenes should restore the structure of the GUS gene and its expression. Thus, the frequency of HR can be estimated by histochemical staining and determination of the number of blue tissue sectors expressing GUS. Compared with the wild type, *bru1-1* plants had approximately fourfold higher levels of HR (Fig. 4A). Elevated HR was apparent under standard growth conditions without any artificial increase in DNA damage by application of genotoxins. Thus, *bru1-1* carries out HR with an efficiency actually exceeding that of the wild type.

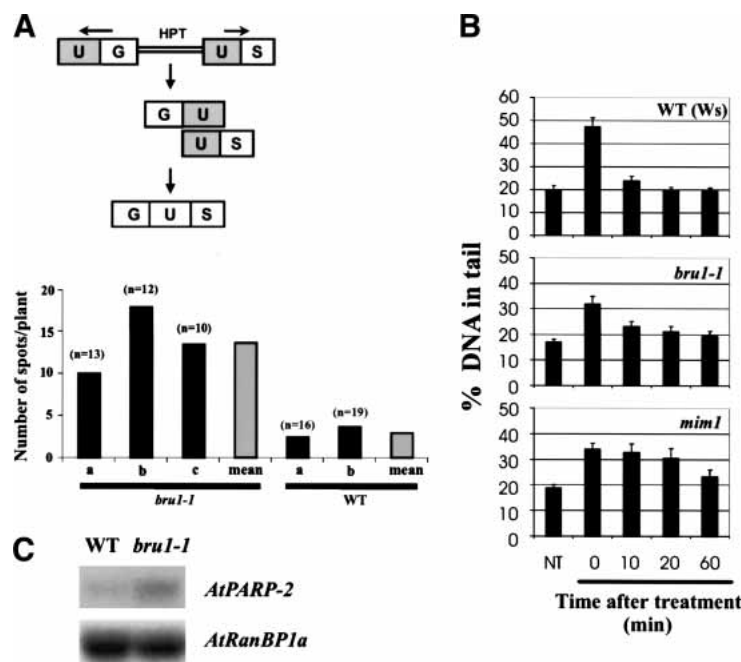
Bru1 is proficient in NHEJ

To investigate whether the sensitivity of *bru1* to high levels of DSBs is due to a deficiency in NHEJ, we assessed repair proficiency of DSBs induced by bleomycin using the comet assay (Fig. 4B). Surprisingly, bleomycin-induced DSBs were repaired in *bru1-1* as rapidly as in the wild type (Fig. 4B). This is in contrast to the *mim1* mutant deficient in a protein related to structural maintenance of chromosome (SMC) proteins (Mengiste et al. 1999) and affected in both HR (Mengiste et al. 1999) and NHEJ (Fig. 4B). These results suggest that *bru1* is proficient in both DSB repair pathways, despite its hypersensitivity to agents inducing additional DNA damage.

Bru1 has constitutively activated genotoxic stress responses

As increased levels of DSBs result in elevated levels of HR in the wild type (Lebel et al. 1993; Puchta et al. 1993, 1996), we hypothesized that *bru1* has naturally increased levels of DSBs and that additional genotoxic treatment with cumulative levels of DSBs would simply exceed the capacity of the repair pathways, possibly leading to an

Figure 4. Frequency of intrachromosomal homologous recombination, DSB-repair proficiency, and constitutive induction of a genotoxic stress responsive gene in *bru1*. (A, top) The recombination trap containing defective but overlapping sequences of the GUS reporter gene separated by a hygromycin phosphotransferase (HPT) marker gene as indicated. Homologous recombination generates a functional GUS reporter gene. The recombination trap was introduced into a *bru1-1* heterozygous plant by crossing, and its F2 progeny was genotyped for individuals homozygous for *bru1-1* or *BRU1* and homozygous for *GUS* transgene. (Bottom) Progenies of three mutant (columns a–c) and two wild-type (columns a and b) parental lines were examined for recombination frequency based on average numbers of GUS sectors per plant. The average scores in all *bru1-1* and wild-type lines (mean) and the numbers of individuals tested from each parental line (n) are indicated. (B) Time course of DSB repair after bleomycin treatment in *bru1*. DSBs induced by bleomycin were detected in a comet assay. Levels of DSBs are given as percent DNA in a tail of comet images. Each experimental point is represented by the mean value and standard error from at least three independent experiments in which 100 comets on four gels were evaluated. (NT) not treated with bleomycin. (C) RNA blot analysis of *AtPARP-2* mRNA. Aliquots (20 μ g) of total RNA from wild type (WT) and *bru1-1* were loaded and hybridized with *AtPARP-2*- and *AtRanBP1a*-specific probes as indicated.



apoptotic-like response. As a consequence, constitutively increased levels of DSBs in *bru1* should be reflected, even under normal growth conditions, by constitutive induction of genotoxic stress responses. To test this hypothesis, we examined the expression of the *Arabidopsis* poly (ADP-ribose) polymerase-2 gene (*AtPARP-2*), a transcriptionally induced marker gene associated with elevated levels of DSBs (Doucet-Chabeaud et al. 2001; Chen et al. 2003). Consistent with the hypothesis, expression of *AtPARP-2* is significantly (two- to three-fold) up-regulated in both *bru1-1* and *bru1-2* plants compared with the wild type and under normal growth conditions (Fig. 4C; data not shown).

Release of transcriptional gene silencing in *bru1* mutants

The *bru1-2* allele was isolated in a screen for mutations interfering with the maintenance of TGS at a silent *LUC* locus (Fig. 5A). In *bru1-2*, silencing of *LUC* is released in leaves of the mutant plants; however, this release is not uniform and luciferase activation can be observed in sectors of tissue (Fig. 5A). These sectors probably reflect stochastic deregulation of silencing followed by mitotic transmission of the newly acquired active state. TGS mutants, such as *mom1* (Amedeo et al. 2000), *met1* (Saze et al. 2003), and *ddm1* (Jeddeloh et al. 1998) release TGS rather uniformly throughout the entire plant (Fig. 5B). To examine whether patchy release of silencing is characteristic of *bru1* mutations and not simply a peculiarity of the transgenic *LUC* locus, we crossed the *bru1-1* mutant

allele to a line carrying transcriptionally silent *GUS* transgene (line 6B5; Morel et al. 2000). In subsequent analysis of the segregating F2 progeny for release of *GUS* expression, only *bru1-1* homozygotes released silencing at the *GUS* transgene (Fig. 5B; data not shown). Importantly, activation of the *GUS* locus occurred in a stochastic fashion (Fig. 5B). Therefore, the patchy release of silencing seems to be an idiosyncrasy of *bru1* mutations. Such epigenetic variegation has not been reported for any other silencing mutant, but *fasciata* (*fas*) mutants show a similar phenotype (H. Kaya, T. Araki, and K. Shibahara, unpubl.). In addition, *bru1-1* plants were examined for the release of silencing at pericentromeric repeats (Fig. 5C), which are transcriptionally silent in wild-type *Arabidopsis* but activated in a number of mutants affected in TGS maintenance (TSI [transcriptionally silent information]; Steimer et al. 2000; Jackson et al. 2002; Saze et al. 2003). Again, only *bru1-1* homozygotes, but not heterozygous or wild-type segregants, released silencing of TSI. Interestingly, the level of TSI expression varied between homozygous *bru1-1* plants, likely reflecting the stochastic nature of silencing release. Variable release of TSI silencing was also observed in homozygous mutants of the other two *bru1* alleles (data not shown).

The *bru1* mutation does not affect global DNA methylation

Because TGS in plants is often associated with DNA hypermethylation and its release with a decrease in methylation (Martienssen and Colot 2001), we examined

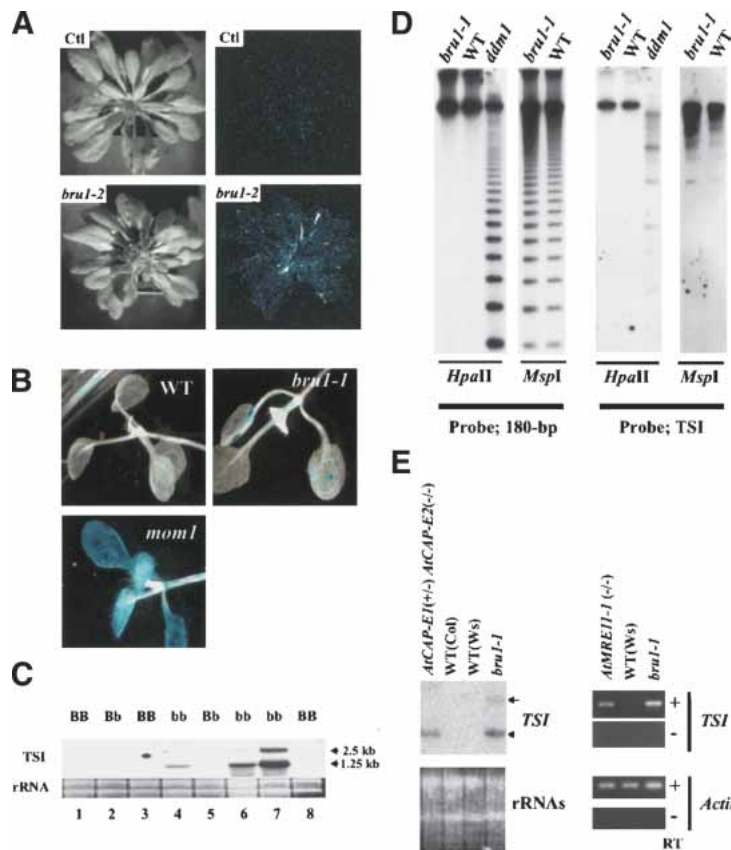


Figure 5. Maintenance of TGS is compromised in *bru1*. (A) *bru1-2* releases silencing of a transcriptionally repressed *LUC* locus. Expression of *LUC* was examined in wild-type (Ctl) and *bru1-2* backgrounds (left column shows bright-light image, right column shows luciferase signals in false-color image). (B) Release of silencing at a transcriptionally silent *GUS* locus in *bru1-1* or *mom1-1* homozygous background and wild-type control. Expression of *GUS* is apparent as blue staining. (C) *bru1* activates transcriptionally silent pericentromeric repeats (TSI). (Upper panel) Segregated homozygous *bru1-1* (bb, lanes 4,6,7), heterozygous (Bb, lanes 2,5), and wild-type (BB, lanes 1,3,8) individuals were tested for expression of TSI (Steimer et al. 2000) by RNA blot analysis. (Lower panel) Ethidium bromide (EtBr)-stained gel before blotting as loading control. (D) *bru1-1* does not affect HpaII and MspI cytosine methylation at the 180-bp centromeric repeats nor at reactivated TSI loci. Genomic DNA was digested with HpaII and MspI and examined by Southern blot analysis with a probe specific for 180-bp centromeric repeats (Vongs et al. 1993) or with a TSI cDNA (Steimer et al. 2000). The mutant *ddm1-7* (Vongs et al. 1993) served as a control. (E) Expression of TSI RNA in seedlings of the double mutant heterozygous for a mutation in the *AtCAP-E1* gene and homozygous for a mutation in the *AtCAP-E2* gene (Siddiqui et al. 2003), and of the homozygous *mre11-1* mutant (Bundock and Hooykaas 2002), analyzed by Northern blot as in C (left), and by RT-PCR (right), with (+) or without (-) reverse transcriptase (RT). RNA levels were compared with the corresponding wild-type and *bru1-1* seedlings. EtBr staining of ribosomal RNAs (rRNA) and RT-PCR of *Actin2* mRNA were used as standards.

the effect of *bru1* mutations on the normally hypermethylated 180-bp centromere repeats (Vongs et al. 1993). We performed Southern blot analysis after restriction with HpaII and MspI, which both recognize the sequence CCGG. HpaII restriction is inhibited by methylation of either of the two cytosines of the recognition site, whereas MspI is inhibited only by methylation of the first cytosine. As shown in Figure 5D, no differences in digestion patterns were detected between wild type and *bru1-1*, indicating that DNA methylation in either CpG or CpNpG sequences is not significantly affected by the *bru1* mutation. This is in sharp contrast to the *ddm1* mutation, which clearly affects DNA methylation levels at the centromeric repeats (Fig. 5D; Vongs et al. 1993; Mittelsten Scheid et al. 1998). Therefore, *bru1* mutations release TGS via a mechanism not associated with global changes in DNA methylation levels. Moreover, it is possible that TGS release is also not accompanied by local changes in DNA methylation, because HpaII and MspI site methylation of TSI loci was not detectably altered despite their transcriptional activation in *bru1-1* (Fig. 5D).

Relationship of *bru1* to other mutants with similar changes in plant morphology

The morphological alterations such as fasciation that are characteristic of *bru1* alleles are reminiscent of *fas1* and *fas2* (Leyser and Furner 1992) or *clavata* (*clv*) mutants

(Clark et al. 1993; Carles and Fletcher 2003). However, the *FAS* and *CLV* genes are thought to function in different pathways (Leyser and Furner 1992; Kaya et al. 2001). *fas1* and *fas2* are loss-of-function alleles in genes encoding the p150 and p60 subunits of the CAF-1 complex, respectively (Kaya et al. 2001), whereas *CLV1*, *CLV2*, and *CLV3* seem to encode components of a signal transduction pathway, namely a heterodimeric receptor kinase and its ligand, involved in intercellular communication (Carles and Fletcher 2003). As *BRU1* is a nuclear protein involved in genome stability, its function is more likely to be related to the function of *FAS* than to the *CLV* complex located at the plasmalemma. In addition, whereas *CLV* genes are specific regulators of shoot apical meristem organization, both *BRU1* and *FAS* also affect root development. Interestingly, mutations in *MRE11* (*AtMRE11-1*; Bundock and Hooykaas 2002) or *SMC2* (*AtCAP-E1* and *AtCAP-E2*, encoding core subunits of the condensin complex; Siddiqui et al. 2003) were also reported to produce fasciation in *Arabidopsis*. Both *MRE11* and condensin complexes are likely to be involved in chromatin/DNA replication and S-phase DNA-damage checkpoints of the cell cycle (D'Amours and Jackson 2002; Hagstrom and Meyer 2003). Therefore, we investigated possible links between chromatin/DNA replication, DNA-damage response, TGS and meristem maintenance, by focusing on the relationship between *bru1* and *fas*, as well as *mre11* and condensin mutants.

We tested whether *mre11* and the condensin mutant also affect epigenetic regulation in heterochromatin. Plants homozygous for mutant alleles of *AtMRE11* gene have reduced vigor and exhibit fasciation in later developmental stages (Bundock and Hooykaas 2002). *AtCAP-E1* and *AtCAP-E2* are functionally redundant, and individuals homozygous for both mutant alleles as well as plants with genotype *AtCAP-E1*^{-/-}, *AtCAP-E2*^{+/-} die during embryo development (Siddiqui et al. 2003). However, *AtCAP-E1*^{+/-}, *AtCAP-E2*^{-/-} plants are viable but exhibit fasciation due to meristem disorganization (Siddiqui et al. 2003). Importantly, the *AtCAP-E1*^{+/-}, *AtCAP-E2*^{-/-} double mutants express TSI at levels similar to *bru1* (Fig. 5E). Furthermore, seedlings homozygous for the *AtMRE11-1* mutation also express TSI, but at a level lower than in *bru1* (Fig. 5E). These results suggest that defects in the S-phase DNA-damage checkpoint or inaccuracies during chromatin/DNA replication cause instability of epigenetic states of newly replicated chromatin, and that this is reflected by the fasciation phenotypes common to the mutants examined. These possibilities were further supported by defects in *fas* mutants, strikingly similar to those observed in *bru1*, as described below.

With regard to the sensitivity to genotoxic stress, both *fas1* and *fas2* were sensitive to 50 ppm of MMS, whereas wild-type plants tolerated 100 ppm (Fig. 6A). Thus, *fas1* and *fas2* are also hypersensitive to genotoxic stress, but to a lower extent than *bru1* (Fig. 6B).

To investigate whether *fas* mutations interfere with TGS, we examined expression of TSI and reactivation of the *GUS* marker, which is silenced within the line 6b5, in the *fas1* and *fas2* background. Similarly to *bru1-1*, TSI was expressed in both *fas1* and *fas2* (Fig. 6D), and crosses between *fas1* or *fas2* and line 6b5 segregated *fas* mutant plants stochastically expressing *GUS* (data not shown). Moreover, transcription of the *CACTA* transposable element (Miura et al. 2001), which is silent in wild-type plants but activated in the *ddm1* mutant, was also found to be stochastically up-regulated in a small proportion of cells of *fas* mutants (H. Kaya, T. Araki, and K. Shibahara, unpubl.). As in *bru1*, release of TGS in *fas1* and *fas2* was not accompanied by global changes in DNA methylation (data not shown).

In both *fas* mutants, shoot apical meristems are disorganized, and this is reflected by distorted meristematic localization of *WUSCHEL* (*WUS*) mRNA (Kaya et al. 2001). *WUS* is required for maintenance of stem cell identity in the shoot apical meristem (Mayer et al. 1998). Similar to *fas1* and *fas2*, disorganized meristems and an abnormal, dispersed expression pattern of *WUS* mRNA were observed in *bru1* (Fig. 6C).

We also examined a potential epistatic relationship between *bru1-1*; *fas1-1* and *bru1-1*; *fas2-2* alleles by constructing double mutant lines. Both double mutants were viable and showed a range of phenotypes similar to those of either of the single parental mutants (Fig. 2; data not shown). Moreover, double mutants showed a level of sensitivity to MMS similar to that of single *bru1-1* mutant plants (Fig. 6B). Thus, by these criteria, *bru1* is most

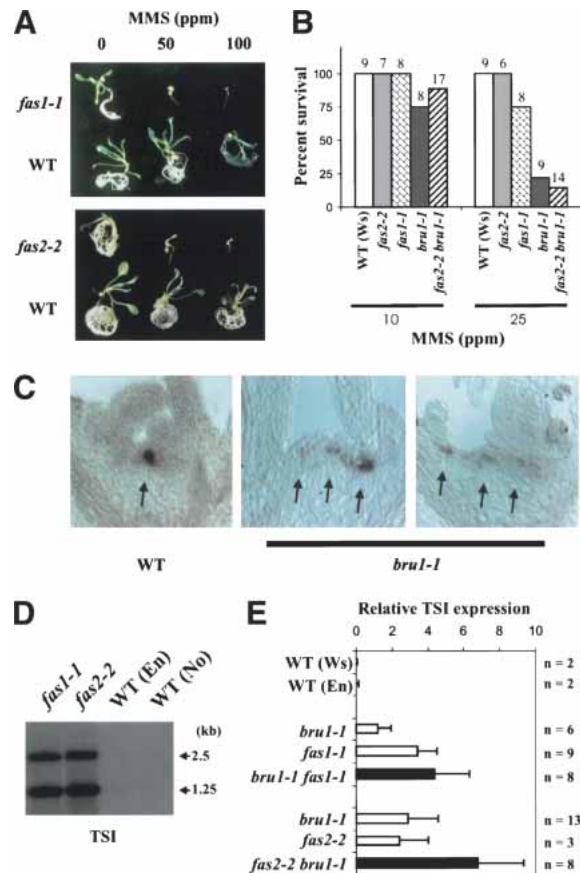


Figure 6. Functional similarity between *bru1* and *fas1* or *fas2* mutants. (A) Sensitivity of *fas1-1* and *fas2-2* and their respective wild types En and No (WT) to MMS. Ten seedlings were tested in each treatment, and representative seedlings are displayed. (B) Sensitivity of mutants to low concentrations of MMS. The number of seedlings used for each treatment is indicated above the bars. The survival rates after each treatment are shown. (C) Distorted shoot apical meristem and misexpression of the *WUSCHEL* gene in *bru1-1* (middle and right) compared with wild type (left). Localization of *WUS* mRNA in shoot apical meristems of wild type and *bru1-1* is visible as dark purple signals (arrows). (D) *fas1-1* and *fas2-2* activate transcriptionally silent pericentromeric repeats (TSI). An RNA blot with equal loading of total cellular RNA (as verified by EtBr-staining of rRNAs) was hybridized with the TSI-specific probe as in Figure 5C. (E) Quantification of TSI expression in *bru1/fas* double mutants. Samples of total RNA of wild type (WT), *bru1-1*, *fas1-1*, *fas2-2*, and their double mutants were analyzed by RNA blotting with a probe specific for TSI and *AtRanBP1a*. Means and standard deviations of TSI RNA levels relative to *AtRanBP1a* mRNA are shown. The numbers of plants tested (n) are indicated. Expression levels in the double mutants were compared to single mutants segregated from two parental lines (middle three and bottom three).

likely to be epistatic to *fas1* and *fas2*. To test whether *BRU1* interacts directly with *CAF-1*, an in vitro protein interaction assay was employed, using a Baculovirus-expressed *BRU1* protein tagged with GST and in vitro-translated subunits of *CAF-1*, which were previously shown to form an active complex (Kaya et al. 2001).

However, we were unable to detect specific interaction between BRU1 and the CAF-1 subunits (data not shown). Surprisingly, double mutants exhibit an additive effect on the release of TGS, reflected by elevated levels of TSI expression (Fig. 6E). Taken together, these results suggest that BRU1 is involved in chromatin assembly, and that BRU1 and CAF-1 contribute additively to postreplicative stability of epigenetic states.

We examined the effects of the *bru1*, *fas1*, and *fas2* mutations on heterochromatin organization in interphase nuclei by fluorescence in situ hybridization (FISH) with a probe specific for centromeric repetitive DNA (Vongs et al. 1993). The analysis revealed altered heterochromatin distribution patterns in *bru1*. Although the organization of centromeric heterochromatin in the majority of *bru1-1* nuclei is similar to wild type (Fig. 7A,C), some nuclei showed a significant diffusion of chromocenters (Fig. 7B). Similarly, decondensation of centromeric heterochromatin was observed in some but not all nuclei in *bru1-2* (Fig. 7D) and *bru1-3* (data not shown). The frequency of nuclei with decondensed chromocenters varies between 2% and 8% and is correlated with the severity of the developmental aberrations. Thus, BRU1 is involved in heterochromatin condensation. In contrast, there were no changes in heterochromatin condensation in *fas1* (data not shown) or *fas2* (Fig. 7E) compared with wild type (Fig. 7E). Double mutants for *bru1* and *fas1* were not different in centromeric heterochromatin organization relative to the *bru1* single mutant (data not shown).

Discussion

We have isolated and characterized an allelic series of mutations in the *BRU1* gene of *Arabidopsis* that result in a drastic increase in sensitivity to genotoxic stress,

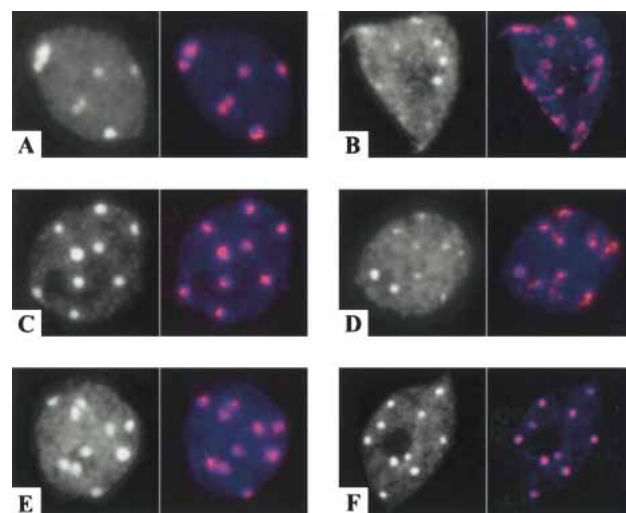


Figure 7. Chromatin organization in interphase nuclei of *bru1* and *fas2* mutants. DAPI-stained nuclei (left panels) and FISH with a probe specific for 180-bp centromeric repeats (right panels) visualizing the compaction of the centromeric DNA (red) in wild-type Ws (A), *bru1-1* (B), wild-type Col (C), *bru1-2* (D), wild-type No (E), and *fas2-2* (F).

elevated levels of intrachromosomal homologous recombination, and instability in the maintenance of TGS. Considering that other previous *Arabidopsis* TGS mutants, such as *ddm1* (Schaeffer et al. 1993; Vongs et al. 1993; Mittelsten Scheid et al. 1998), *mom1* (Amedeo et al. 2000), and *met1* (Kankel et al. 2003; Saze et al. 2003), have unchanged resistance to DNA-damaging treatments (data not shown), BRU1 seems to play a dual function as guardian of both genetic and epigenetic information.

BRU1 encodes a novel protein. Clues to BRU1 function were provided by comparative analysis of *bru1* with mutants in CAF-1 subunits, MRE11, or condensin subunits. These mutations result in developmental alterations similar to *bru1*, and the corresponding genes have been functionally related to DNA and chromatin replication. Interestingly, these complexes are thought to be important for the replication of chromosomal regions containing repeated, transcriptionally silent DNA packaged into heterochromatin (Enomoto and Berman 1998; D'Amours and Jackson 2002; Lobachev et al. 2002; Hagstrom and Meyer 2003), whereas *bru1* mutations release gene silencing and alter chromatin structure in the area of pericentromeric heterochromatin.

We substantiated the phenotypic correlations by demonstrating that *fas1* and *fas2* mutants were also hypersensitive to MMS, although less so than *bru1*. This may reflect functional redundancy between CAF-1 (Kaya et al. 2001) and the putative ASF1 chromatin-assembling subunits encoded by genes At5G38110 and At1G66740 in *Arabidopsis*, whereas there is no obvious redundancy for BRU1. It is also possible that BRU1 has further roles in the prevention or the repair of DNA damage.

In the apical meristems of *fas1* and *fas2* mutants, the maintenance of spatial expression patterns of the *WUS* gene is disturbed, and it has been proposed that CAF-1 secures the organization of meristems by stabilization of the epigenetic inheritance of gene expression patterns (Kaya et al. 2001). In *bru1*, *WUS* is also mis-expressed, and the shoot apical meristem structure is disrupted similarly to *fas1* and *fas2*. A common role of BRU1 and CAF-1 subunits in epigenetic inheritance is further supported by the activation of transcriptionally silent pericentromeric repeats in all these mutants. Moreover, this TGS release occurs without obvious changes in DNA methylation and in a variegated fashion, implying related mechanisms of silencing control. Importantly, epistatic analyses suggested that these proteins serve both overlapping and nonoverlapping functions.

Although no direct interaction was detected between BRU1 and the CAF-1 subunits or histones, BRU1 may be indirectly involved in histone chaperoning mediated by CAF-1 or associated with CAF-1 through additional protein(s). In yeast, CAF-1-mediated nucleosome assembly following DNA replication facilitates stabilization of newly reconstituted chromatin (Enomoto and Berman 1998). Considering the alterations in centromeric heterochromatin observed in *bru1*, BRU1 possibly plays a role in chromatin replication and may also be involved in postreplicative stabilization of chromatin structure.

As with the *fas* mutants, *bru1* shares several features beyond phenotypic similarity with mutations in *MRE11* and condensin genes (Bundock and Hooykaas 2002; Siddiqui et al. 2003). *MRE11* is part of the *MRE11/RAD50/NBS* complex that is thought to be involved in DSB repair by NHEJ. Mutations in the individual subunits produce largely divergent phenotypes. Both *mre11* and *rad50* mutants are hypersensitive to treatments provoking DSBs (although not to the extent of the *bru1* mutation; Gallego et al. 2001; Bundock and Hooykaas 2002) and show elevated levels of homologous recombination (in *Arabidopsis* documented only for *rad50*; Gherbi et al. 2001). However, only *mre11* mutants, and not *rad50* mutants, exhibit fasciated phenotypes similar to *bru1*. Thus, deficiency in NHEJ does not inevitably result in developmental abnormalities characteristic of *bru1* and *mre11*. This implies that BRU1 function is unlikely to be linked to NHEJ. Indeed, *bru1* mutants are proficient in NHEJ, as shown by the comet assay. Depletion of the *MRE11* complex in *Xenopus* leads to spontaneous accumulation of DSBs (Costanzo et al. 2001). Considering the similarity of *bru1* and *mre11* mutants in *Arabidopsis*, it is possible that hypersensitivity of *bru1* to genotoxic treatments is in part a result of constitutively elevated levels of DSBs, prior to induction of additional DNA DSBs by exogenously applied genotoxins. This is in agreement with the constantly elevated expression level in *bru1* of *AtPARP-2*, a marker gene associated with elevated levels of DNA damage (Doucet-Chabeaud et al. 2001; Chen et al. 2003). In addition, consistent with the spontaneous up-regulation of genotoxic stress response, we observed less damage in *bru1* than in wild type shortly after bleomycin treatment (Fig. 4B; data not shown). Importantly, defects in chromatin assembly in human cells induced by a dominant-negative mutation of the CAF-1 p150 subunit result in stalled replication forks that are inappropriately processed and lead to increased DSBs (Ye et al. 2003). This is accompanied by activation of the S-phase checkpoint, resulting in S-phase arrest (Ye et al. 2003). RNAi depletion of CAF-1 also causes S-phase checkpoint activation and accumulation of cells in early and mid-S phase. In this case, the arrest seemed to be a consequence of a perturbation in chromatin replication (Hoek and Stillman 2003), because the ATR (ATM/Rad3-related) signaling kinase but not the ATM (ataxia-telangiectasia-mutated) pathway was activated. The existence of a plant homolog of the mammalian ATM kinase (Garcia et al. 2003), a key mediator of S-phase checkpoints and activation of concomitant DNA-repair responses, suggests that a similar genome surveillance system could operate in plants. It is possible that chromatin replication in the absence of functional BRU1 protein causes instability of the replication forks, leading either to elevated DSBs or to deficiencies in chromatin replication. In both cases, this results in activation of the S-phase checkpoint control.

Mutations in condensin and *mre11*, but not in *rad50* genes, result in developmental abnormalities similar to *bru1*. Noticeably, both condensin and *MRE11* are involved in S-phase checkpoint activation (Aono et al.

2002; D'Amours and Jackson 2002; Hagstrom and Meyer 2003). Thus, it is plausible that BRU1 also plays a role in this process together with the CAF-1 complex, acting upstream of the checkpoint activation. Interestingly, the most crucial roles assigned to *MRE11* and CAF-1 are for the smooth and high-fidelity replication of DNA repeat regions, which are usually packaged into compact heterochromatin (Lobachev et al. 2002; Hoek and Stillman 2003). In the present study, we demonstrate that the silencing of TSI is also compromised in *mre11* and condensin mutants. The similar developmental abnormalities and destabilization of the genetic and epigenetic inheritance observed in all mutants examined here emphasize the tight link between chromatin assembly and the S-phase checkpoint. One possibility is that inaccurate chromatin replication and/or checkpoint defects can trigger downstream events such as accumulation of DNA damage, aberrant chromatin structures, and release of epigenetic gene silencing. Obviously, all of these events can also take place during unscheduled DNA synthesis accompanying DNA repair, and deficiencies in the components involved may contribute to the postreplicative increase of DNA damage, thus exhausting its efficient repair.

In summary, we propose that the novel BRU1 protein is involved in structural and functional stabilization of chromatin. Complex and multiple facets of *bru1* mutants and the overlapping roles of BRU1, CAF-1, *MRE11*, and condensin strongly suggest that BRU1 is a new molecular link between maintenance of both genetic and epigenetic information and the control of development.

Materials and methods

Plant material and growth conditions

For isolation of *bru1-1* and *bru1-2*, we screened 2500 T-DNA-mutagenized *Arabidopsis* (ecotype Wassilevskija [Ws]) lines, generated at the Institute de la Recherche Agronomique (INRA), Versailles, France, and 50,000 lines of *Arabidopsis* (ecotype Columbia [Col]) containing a transcriptionally silent *LUC* locus mutagenized by EMS. *bru1-3* (ecotype Col) was obtained from the Torrey Mesa Research Institute (<http://www.tmri.org>). *fas1-1* (ecotype Enkheim [En]) and *fas2-2* (ecotype Nossen [No]) were described previously (Leyser and Furner 1992; Kaya et al. 2001). The genotypes of *bru1* and *fas* mutants were determined by PCR with primers differentiating between wild-type and mutated loci. The line 6b5 (ecotype Col) containing transcriptionally silent, multiple copies of a *GUS* transgene (Morel et al. 2000) was kindly provided by H. Vaucheret (INRA, Versailles, France). Plants were grown in 12 h light/12 h dark cycles (short day) or 16 h light/8 h dark cycles (long day) at 21°C during the day and 16°C during the night. Seedlings of mutants homozygous for *AtMRE11-1* (ecotype Ws) and the double mutant for *AtCAP-E1^{+/-}* (ecotype Ws); *AtCAP-E2^{-/-}* (ecotype Col) were grown and selected on half-strength MS medium supplemented with 10 g/L sucrose, 1.5% agar, and 50 mg/L kanamycin in long-day conditions as described (Bundock and Hooykaas 2002; Siddiqui et al. 2003).

Tests for sensitivity to genotoxic stresses

Three-day-old seedlings grown under aseptic conditions were tested for sensitivity to MMS (Fluka) as described (Revenkova et

al. 1999). Sensitivity to bleomycin, mitomycin C, and UV-C were examined as described [Mengiste et al. 1999].

Map-based cloning of the *BRU1* gene

bru1- was crossed to Col and Landsberg erecta (Ler) wild-type plants. DNA from 1400 F₂ plants was analyzed for SSLP and CAPS markers, generated, and evaluated based on the polymorphism data provided by CEREON (<http://www.arabidopsis.org/Cereon>). Genes at the mapped interval were amplified by PCR and sequenced. A *BRU1* cDNA was amplified by PCR from cDNA mixtures, using primers designed to contain sequences corresponding to 5'- and 3'-ends of the annotated ORF, respectively (The *Arabidopsis* Information Resource, <http://arabidopsis.org>; forward, 5'-TAGGAATTCCTGATGGGTCGA TTAGATGTAGCTGCGGCG-3', and reverse, 5'-TAGGAATT CCTGGTCCTTTCTGCAGCATGATTGACTCCGC-3').

Subcellular localization of *BRU1*-GFP fusion protein

The *BRU1* cDNA was inserted after sequence determination into a plant expression vector (derivative of pUC-based vector) containing a reporter gene encoding enhanced GFP (F64L, S65C) and driven by a modified CaMV 35S promoter. The resulting plasmids were introduced into *Nicotiana plumbaginifolia* protoplasts by PEG-mediated transformation, and the protoplasts were incubated at 28°C in the dark for 8 h. GFP signals were analyzed using a Leitz DMR fluorescence microscope, and images were captured with a SPOT RT camera (Diagnostic Instruments) using the SPOT advanced software. For visualization of nuclei, protoplasts were stained with 10 mg/mL DAPI in the presence of 0.5% Triton X-100.

Southern and Northern blot analyses, RT-PCR, fluorescence in situ hybridization, and in situ mRNA localization

Southern and Northern blot analyses were performed and specific probes for TSI and *AtRanBP1a* were prepared as described [Steimer et al. 2000]. A cDNA probe for *AtPARP-2* was obtained by RT-PCR as described [Doucet-Chabeaud et al. 2001]. RT-PCR with poly-dT primer for cDNA synthesis and with *Actin2*- and TSI-specific primers for cDNA amplification was performed as described [Saze et al. 2003]. For fluorescence in situ hybridization, the biotin-labeled 180-bp centromeric repeat [Vongs et al. 1993] was hybridized to nuclear spreads prepared from ethanol-acetic acid-fixed rosette leaves, detected with Texas Red conjugated avidin [Mittelsten Scheid et al. 2002; Probst et al. 2003], and analyzed using a Leitz DMR fluorescence microscope. For each genotype, at least five plants were examined and 150–350 nuclei per plant were scored. In situ localization of *WUS* mRNA was performed as described [Kaya et al. 2001].

GUS staining and intrachromosomal recombination assay

bru1-1 was crossed to line 651 (ecotype C24) carrying a reporter construct for intrachromosomal recombination [Swoboda et al. 1994]. Four-week-old F₃ plants homozygous for the mutant *bru1-1* allele or wild-type *BRU1* gene and homozygous for the *GUS* transgene were stained histochemically as described [Amedeo et al. 2000].

Comet assay

A single-cell gel electrophoresis (comet) assay to detect DSBs [N/N protocol] was performed as described [Angelis et al. 1999, 2000], using cells from aerial tissues of 10-day-old seedlings.

DSB repair was measured after induction of DSBs by treatment with bleomycin (30 µg/mL) for 1 h.

Luciferase assay

Plants carrying the transcriptionally silent *LUC* locus were sprayed with a 1-mM aqueous solution of the substrate luciferin. Luciferin (Molecular Probes) was dissolved in sterile water and stored frozen as a 25-mM stock solution. In vivo imaging of luciferase activity was performed with a CCD camera system employing Argus 50 Software [Hamamatsu Photonic Deutschland].

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Note added in proof

While this manuscript was under review, Guyomarc'h et al. (2004) and Suzuki et al. (2004) reported isolation of additional mutant alleles of the *BRU1* gene causing similar developmental abnormalities as described here. These data complement and confirm the phenotypic description of *bru1* mutants given here.

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